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Molecular determinants of apoptosis induced by cytotoxic drugs

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Summary

Recent experimental evidence suggests that apoptosis pathways such as the CD95 system are an important mediator of chemotherapy-induced apoptosis in various tumor cell lines. Therapeutic concentrations of cytotoxic drugs induce CD95 and CD95-L that mediates apoptosis via an autocrine/paracrine loop by crosslinking CD95. Interfering with CD95-L/receptor interaction by antagonistic antibodies to the receptor or by inhibition of CD95-L expression strongly reduces apoptosis. Drug-induced apoptosis critically depends on activation of caspases since apoptosis is almost completely abrogated by the caspase inhibitor zVAD-fmk. The receptor apical caspase FLICE/MACH (caspase-8) and the downstream caspase CPP32 (caspase-3) are cleaved resulting in processing of substrates such as the nuclear enzyme PARP. In addition, the response to cytotoxic drugs is modulated by pro- and antiapoptotic proteins of the Bcl-2 family and p53. Defects in apoptosis pathways, e.g. deficient upregulation of CD95-L, downregulation of CD95 expression or blockade of caspase activation may confer resistance to cytotoxic drug treatment. Thus, chemosensitivity of tumor cells depends on intact apoptosis pathways such as the CD95 system that are activated by chemotherapeutic drugs. These findings may have implications for drug sensitivity and resistance of tumor cells.

Key words apoptosis – CD95 – drugs

Molekulare Grundlagen der Zytostatika-induzierten Apoptose

Neuere Daten weisen darauf hin, daß Apoptosesignalwege wie das CD95 System eine wichtige Rolle spielen als Vermittler von Zytostatika-induzierter Apoptose in verschiedenen Tumorzellinien. Therapeutische Dosen von Zytostatika induzieren CD95 und CD95-L, der auf autokrinem oder parakrinem Weg über Trimerisierung von CD95 Apoptose auslöst. Durch Verhinderung der Interaktion von CD95-L und CD95 durch antagonistische Antikörper oder durch Blockade der CD95-L Bildung wird das Ausmaß der Apoptose stark reduziert. Zytostatika-induzierte Apoptose bedarf der Aktivierung von Caspasen, da Apoptose durch den Caspaseninhibitor zVAD-fmk nahezu vollständig verhindert wird. Die Rezeptor-apikale Caspase FLICE/MACH (Caspase-8) und die distale Caspase CPP32 (Caspase-3) werden gespalten, was zur Spaltung von Substraten wie dem nuklearen Enzym PARP führt. Die Zytostatikawirkung wird ausserdem moduliert durch pro- und antiapoptotische Proteine der Bcl-2 Familie und p53. Defekte in Apoptosesignalwegen, z.B. fehlende Hochregulation von CD95-L, Herunterregulation der CD95 Expression oder Blockade der Caspasenaktivierung können zur Zytostatikaresistenz beitragen. Chemosensitivität von Tumorzellen hängt infolgedessen von intakten Apoptosesignalwegen wie dem CD95 System ab, die durch Zytostatika aktiviert werden. Diese Ergebnisse sind von Bedeutung für Chemosensitivität und Resistenz von Tumorzellen.

Schlüsselwörter Apoptose – CD95 – Zytostatika

Introduction

Chemotherapeutic agents irrespective of their intracellular target have been reported to act primarily through induction of apoptosis in sensitive cancer cells (1). Although extensive studies of the biochemical and molecular pharmacology of drug-target cell interaction have been performed, the precise molecular requirements by which cytotoxic drugs initiate apoptosis pathways are poorly defined.

Cell surface receptor molecules of the tumor necrosis factor/ nerve growth factor receptor superfamily such as CD95 are involved in regulation of apoptosis (2–5). CD95 is a 45 kDa type I transmembrane receptor expressed on a variety of normal and neoplastic cells (3). Following crosslinking of CD95 either with its specific ligand or with an agonistic anti-CD95 antibody, a death signal is generated in susceptible cells that catalyzes cleavage of the caspase cascade of cysteine proteases leading to proteolysis of substrates such as the nuclear enzyme PARP and ultimately to cell death (5–7). CD95 ligand (CD95-L) is a 40 kDA type II transmembrane molecule of the tumor necrosis factor/ nerve growth factor family of ligands which may also occur in a soluble form released from the cell surface by proteolytic cleavage (8, 9).

Although the key regulatory role of the CD95 system has mostly been studied within the immune system (10), there

Klin. Pädiatr. 210 (1998) 148–152

The abbreviations used are: CD95-L, CD95 ligand; Doxo, doxorubicin; ICE, interleukin 1β-converting enzyme; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

is mounting evidence that activation of key apoptosis systems such as CD95 may also contribute to the cytotoxic effect of drugs used in the treatment of leukemia and solid tumors (11–17). In the present report, we describe some of the molecular determinants of drug-induced apoptosis .

Materials and Methods

Drugs. Doxorubicin (Farmitalia, Milano, Italy) was provided as pure substances and dissolved in sterile water prior to each experiment (1mg/ml).

Cell Culture. Neuroblastoma (SHEP), Hodgkin (L540CY), Ewing's sarcoma (A17/95), medulloblastoma (Daoy), glioblastoma (A172), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were cultured in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat inactivated FCS (Conco, Wiesbaden, Germany), 10 mM HEPES, pH 7.3 (Biochrom, Berlin, Germany), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2 mM L-glutamine (Biochrom).

Determination of apoptosis. Cells were plated at a density of 5×10^4 cells/cm^2 and incubated for indicated times with doxorubicin. Cells were harvested by trypsinization using 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+} (Life Technologies, Inc.). Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide stained nuclei as previously described (18) using CELLQuest software (Becton Dickinson, Heidelberg, Germany).

RT-PCR for CD95-L mRNA. Total RNA was prepared using the Qiagen total RNA kit (Qiagen, Hilden, Germany). RNA was converted to cDNA by reverse transcription and amplified for 38 cycles by PCR in a thermocycler (Stratagene, Heidelberg, Germany) using the Gene Amplification RNA-PCR kit (Perkin Elmer, Branchburg, NJ) following the manufacturer's instructions. A 500-base pair fragment of CD95-L was amplified using primer 5'ATGTTTCAGCTCTTCCACCTACAGA3' and 5'CCAGAGAGAGCTCAGATACGTTGAC3'. Expression of b-actin (MWG-Biotech, Ebersberg, Germany) was used as a standard for RNA integrity and equal gel loading. PCR-reaction products were run at 60 V for 2 h on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination.

Western blot analysis. Cells were lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva, Heidelberg, Germany) and 1mM PMSF (Sigma, Deisenhofen, Germany) followed by high-speed centrifugation. Membrane proteins were eluted in buffer containing 0.1 M glycine, pH 3.0 and 1.5 M Tris, pH 8.8. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL). 40 µg protein per lane was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of CD95-L, FLICE, CPP32, PARP, Bax, Bcl-x, Bcl-2 and p53 protein was done using mouse anti-CD95-L monoclonal antibody (1:5000, Transduction Laboratories, Lexington, KY), mouse anti-FLICE monoclonal antibody C15 (ref. 19; 1:5 dilution of hybridoma supernatant), mouse anti-CPP32 monoclonal antibody (1:1000), rabbit anti-PARP polyclonal antibody (1:10000, Enzyme Systems Products), rabbit anti-Bax polyclonal antibody (1:500, Calbiochem, Bad Soden, Germany), rabbit anti-Bcl-x polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bcl-2 monoclonal antibody (1:1000, Santa Cruz Biotechnology), mouse anti-p53 monoclonal antibody (1:1000, Transduction Laboratories) and goat anti-mouse IgG or goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology). ECL (Amersham) was used for detection.

Results

Cytotoxic drugs have been described to cause cell death by triggering apoptosis in various target cells (1). We previously found that the CD95 system, which is known as a key regulator of the immune system, also mediated drug-induced apoptosis of leukemia and neuroblastoma cells (11–14). Treatment with cytotoxic drugs induced expression of CD95 and CD95-L that triggered apoptosis in an autocrine/paracrine manner by crosslinking CD95 (11–14). Interfering with CD95-L/receptor interaction by using antagonistic antibodies to the receptor or by blocking CD95-L production using cyclosporine A strongly inhibited drug-triggered apoptosis (11–14). In addition, leukemia or neuroblastoma cells which were resistant to APO-1-mediated apoptosis were cross-resistant to cytotoxic drug treatment (20). Vice versa, drug-resistant cells also exhibited cross-resistance to APO-1-mediated apoptosis (20) suggesting that execution of cell death triggered by anti-APO-1 or cytotoxic drugs might require, at least in part, identical molecular effector pathways. During the effector phase of various forms of apoptosis, proteins of the caspase family of cysteine proteases (ICE/Ced-3 like proteases) are activated (21). Recent data from our laboratory indicate that sensitivity for drug-induced cell death critically depends on cleavage of caspases (22). Figure 1 demonstrates activation of different levels of the caspase cascade upon treatment of neuroblastoma cells with doxorubicin. The receptor proximal caspase FLICE was cleaved to the p43 and p41 cleavage intermediates and the p18 active subunit and the downstream caspase CPP32 was processed to the p17 ac-

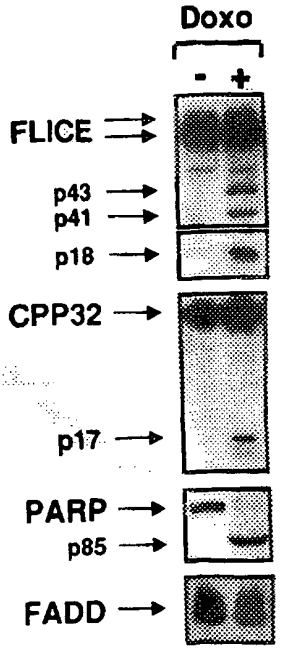

Fig. 1 Doxorubicin-induced activation of caspases. SHEP cells were treated (+) with 0.5 µg/ml doxorubicin for 24 h. 40 µg protein per lane isolated from cell lysates were separated by 15% SDS-PAGE. Immunodetection of FLICE, CPP32, PARP and FADD protein was performed by mouse anti-FLICE monoclonal antibody, mouse anti-CPP32 monoclonal antibody, rabbit anti-PARP polyclonal antibody or mouse anti-FADD monoclonal antibody and ECL

tive subunit resulting in cleavage of the nuclear enzyme PARP to its characteristic p85 fragment (Figure 1). No difference was seen in expression levels of the adaptor molecule FADD (23) which couples CD95 to proteins of the caspase family (Figure 1). Cleavage of the receptor apical caspase FLICE following drug treatment further supports the concept that anticancer agents cause cell death by inducing CD95-L expression that triggers the CD95 signaling pathway by crosslinking CD95 thereby initiating activation of FLICE. In addition to leukemia and neuroblastoma cells, induction of CD95-L and cleavage of caspases upon drug treatment was also found in a variety of other chemosensitive human tumor cell lines including Hodgkin, Ewing's sarcoma, medulloblastoma, glioblastoma and colon carcinoma cells, but not in cells which poorly responded to anticancer agents (Figure 2, Table 1) suggesting that chemosensitivity of tumor cells was related to intact apoptosis pathways and activation of the CD95 system.

In addition to the CD95 system, response to cytotoxic drugs may be modulated by numerous mechanisms including Bcl-2 related proteins or p53 (24, 25). Treatment of neuroblastoma cells with doxorubicin resulted in upregulation of the proapoptotic protein Bax without any change in expression levels of the antiapoptotic proteins Bcl-2 or Bcl-x_L thereby changing the ratio of death agonists to antagonists in favor of apoptosis (Figure 3A). Wild-type p53, known as

Tab. 1 Doxorubicin-induced apoptosis. Neuroblastoma (SHEP), Hodgkin (L540CY), Ewing's sarcoma (A17/95), medulloblastoma (Daoy), glioblastoma (A172), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7), renal cell carcinoma (KTCTL-26) cells were treated with 0.5 µg/ml doxorubicin for 72 h. Apoptosis was assessed by FACS analysis of propidium iodide stained nuclei. Percentage of specific apoptosis was calculated as follows: 100 x [experimental apoptosis (%) – spontaneous apoptosis (%)]/[100% – spontaneous apoptosis (%)]. Data are the mean of triplicates ± standard deviations. Similar results were obtained in three separate experiments

Cell line	% Apoptosis
SHEP	86.86 ± 2.96
L540CY	82.3 ± 4.83
A17/95	89.39 ± 4.14
Daoy	89.48 ± 3.52
A172	47.37 ± 1.79
HT-29	63.16 ± 2.11
H-147	27.52 ± 4.28
MCF-7	5.88 ± 0.93
KTCTL-26	11.72 ± 1.24

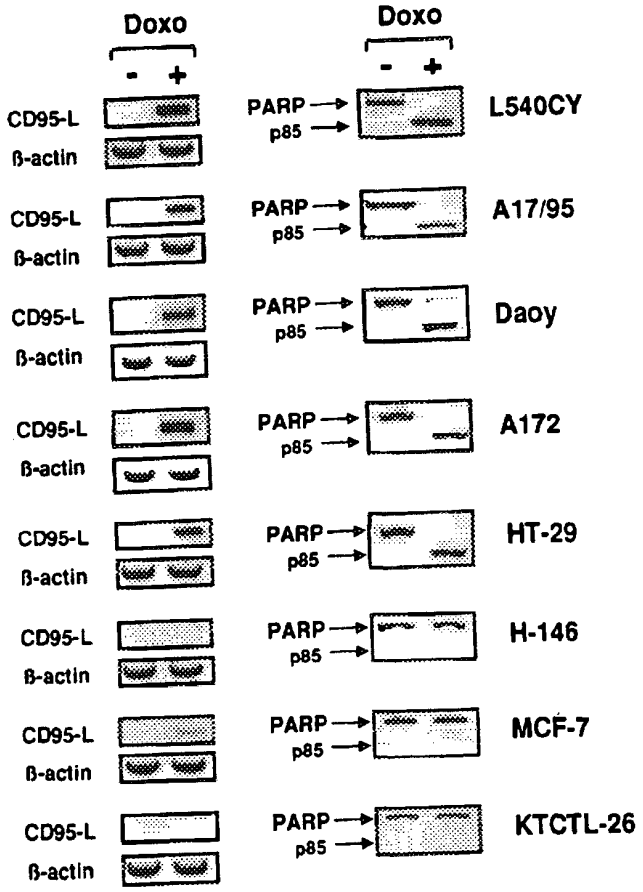

Fig. 2 Doxorubicin-induced CD95-L mRNA expression and PARP cleavage in various tumor cells.
Hodgkin (L540CY), Ewing's sarcoma (A17/95), medulloblastoma (Daoy), glioblastoma (A172), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml doxorubicin for 24 h. CD95-L mRNA expression was determined by RT-PCR. Expression of b-actin was used to control RNA integrity and equal gel loading. Western blot analysis was performed as described in Figure 2

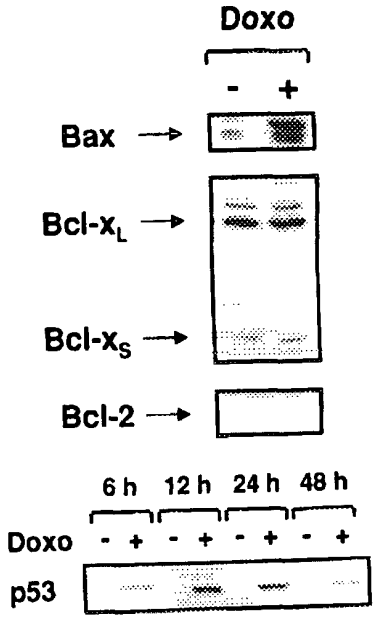

Fig. 3 Doxorubicin-induced expression of Bcl-2 related proteins and p53.
A, Induction of Bax protein. SHEP cells were treated (+) with 0.5 µg/ml doxorubicin for 24 h. Western blot analysis was performed as described in Figure 2 using rabbit anti-Bax polyclonal antibody, rabbit anti-Bcl-x polyclonal antibody or mouse anti-Bcl-2 monoclonal antibody
B, Accumulation of p53 protein. SHEP cells were treated (+) with 0.5 µg/ml doxorubicin for indicated times. Western blot analysis was performed as described in Figure 2 using mouse anti-p53 monoclonal antibody

"guardian of the genome" which senses DNA damage and which may also act as transcriptional activator of the *BAX* gene (26), accumulated upon drug treatment indicating that p53 might be involved in upregulation of *BAX* gene expression (Figure 3B).

Discussion

Although cytotoxic drugs are considered to cause cell death by triggering apoptosis in susceptible target cells (1), the precise molecular mechanisms are not well defined. In the present report, we have demonstrated some of the molecular determinants of drug-induced apoptosis. A summary of our data and similar data published in the literature would suggest the following scenario for drug-induced apoptosis (Figure 4):

Anticancer drugs may initially target diverse cellular functions such as DNA or metabolism. This damage is translated into a cellular response which leads to activation of the apoptotic machinery. Translation may involve p53 or cellular "stress" signaling pathways (27, 28). p53 has been implicated in the transcriptional activation of proapoptotic molecules such as bax or CD95 (26, 29). Common downstream apoptosis pathways involving activation of caspases may be activated through ligand/receptor driven amplifier systems such as the CD95 system, which are comprised of soluble or membrane bound ligands that stimulate their cognate receptors. The receptor proximal caspase FLICE and the downstream caspase CPP32 are cleaved during drug-induced apoptosis resulting in processing of death substrates such as the nuclear repair enzyme PARP and ultimately in cell death. The apoptosis pathway is tightly regulated by pro- and antiapoptotic programs, e.g. proteins of the Bcl-2 family that play a key role in regulation of apoptosis through homo- or heterodimerization (24). The ratio of pro-apoptotic Bcl-2 related proteins such as Bax and Bcl-x_S to anti-apoptotic family members including Bcl-2 and Bcl-x_L dictates the susceptibility of cells to various apoptotic stimuli. Recent data also indicate that overexpression of Bcl-2 or Bcl-x_L, which are localized to intracellular membranes including the mitochondrial membrane, inhibit apoptosis by preventing cytochrome c release from mitochondria to the cytosol thereby inhibiting activation of caspases (30, 31). In fact, the first link between chemosensitivity of tumors and apoptosis regulating molecules was provided by experiments demonstrating increased chemoresistance in cells that overexpress antiapoptotic molecules such as Bcl-2 and Bcl-x_L. Failure to properly activate apoptosis pathways, which may occur at different levels of the signaling cascade, e.g. deficient induction of CD95-L, downregulation of CD95 cell surface expression or defects in caspase activation, may confer resistance to cytotoxic drug treatment (20, 22). However, drug-induced apoptosis may also occur independently of the CD95 system (32, 33).

Taken together, our findings suggest that chemosensitivity of tumor cells depends on intact apoptosis pathways such as the CD95 system that are activated by chemotherapeutic agents. By providing insights into the molecular mechanisms of drug-induced apoptosis these findings may have important implications for the development of novel treatment approaches to increase sensitivity and to overcome resistance of tumor cells.

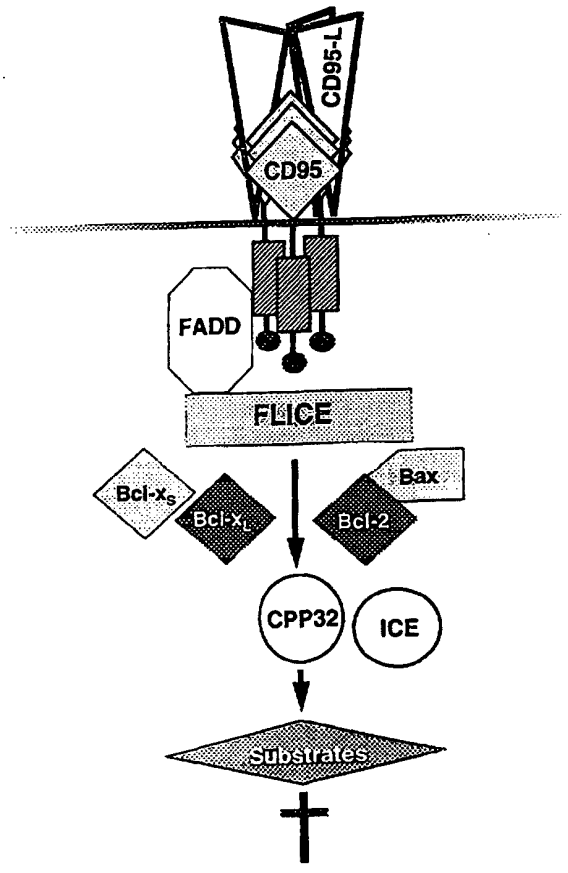

Fig. 4 Hypothetical scenario of drug-induced apoptosis. For details see discussion section

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